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Synthesis and Processing of the Nonstructural Polyproteins of Several Temperature-Sensitive Mutants of Sindbis Virus

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We have examined the synthesis and processing of nonstructural polyproteins by several temperature-sensitive mutants of Sindbis virus, representing the four known RNA-minus complementation groups. Four mutants that possess mutations in the C-terminal domain of nonstructural protein nsP2 all demonstrated aberrant processing patterns when cells infected with these mutants were shifted from a permissive (30°) to a nonpermissive (40°) temperature. Mutants *ts*17, *ts*18, and *ts*24 showed severe defects in processing of nonstructural polyproteins at 40°, whereas *ts*7 showed only a minor defect. In each case, cleavage of the bond between nsP2 and nsP3 was greatly reduced whereas cleavage between nsP1 and nsP2 occurred almost normally, giving rise to a set of polyprotein precursors not seen in wild-type-infected cells at this stage of infection. The nsP1 produced by these mutants was unstable and only small amounts could be detected in infected cells at the nonpermissive temperature. Submolar quantities of nsP2 were also present. We suggest that nsP1 and nsP2 may function as a complex and that free nsP1, and possibly nsP2, is degraded. Cleavage between nsP3 and nsP4 appeared to be normal in the mutants except in the case of *ts*17, where upon shift to 40° P34 was unstable and nsP4 accumulated. We propose that the change in the P34/nsP4 ratio upon shift is responsible for the previously observed temperature sensitivity of subgenomic 26 S RNA synthesis in *ts*17 and for the failure of the mutant to regulate minus strand synthesis at 40°. Other mutations tested, including *ts*21, which is found in the N-terminal half of nsP2, *ts*11, which has a mutation in nsP1, and *ts*6, which has a mutation in nsP4, all demonstrated nonstructural polyprotein processing indistinguishable from that in wild-type-infected cells. These results support our conclusion, based upon deletion mapping studies, that the C-terminal domain of nsP2 contains the nonstructural proteinase activity.

INTRODUCTION

The type alphavirus, Sindbis virus, has a single strand RNA genome of 11.7 kilobases whose sequence is known (Strauss *et al.*, 1984). During infection this plus strand RNA serves as a template for the transcription of full-length minus strand RNA, which in turn serves as a template for the synthesis of both 26 S subgenomic mRNA and additional genomic RNA (reviewed in Strauss and Strauss, 1986; Strauss *et al.*, 1987). The subgenomic mRNA is translated into the structural proteins of the virion, which include the capsid protein and two envelope glycoproteins, whereas the nonstructural proteins of the virus, which are presumed to be involved in viral RNA replication, are translated as two large polyprotein precursors from the 5′ terminal two-thirds of the genomic RNA. The smaller and more abundant polyprotein (~200 kDa) contains the sequences of nsP1, nsP2, and nsP3, while the larger precursor (~250 kDa), which is produced in smaller quantities by readthrough of an opal codon at the end of nsP3, contains, in addition, the sequence of the fourth nonstructural protein, nsP4 (Hardy and Strauss, 1988; Lopez *et al.*, 1985; Strauss *et al.*, 1983). Processing of these precursors occurs by a proteolytic activity within the C-terminal half of nsP2 (Hardy and Strauss, 1989; Ding and Schlesinger, 1989).

Large numbers of temperature-sensitive (*ts*) RNA-minus mutants of Sindbis virus, which are defective in viral RNA synthesis at the nonpermissive temperature, have been isolated and assigned by complementation to four groups (A, B, F, and G) (Burge and Pfefferkorn, 1966a,b; Strauss *et al.*, 1976). Recently, the amino acid changes within the viral nonstructural proteins responsible for the *ts* phenotype of members of each group have been identified, and from this mapping some of the functions of these proteins have been deduced (Hahn *et al.*, 1989a,b). The only mutant in group B, *ts*11, has a mutation in nsP1, implicating this protein in the initiation of minus strand synthesis (Hahn *et al.*, 1989b). This protein has also been found to be involved in methylation of the 5′ terminal cap structure on virion RNAs (Mi *et al.*, 1989). Three group F mutants have been mapped to nsP4 (Hahn *et al.*, 1989a). The best characterized member, *ts*6, fails to synthesize any RNA upon shift from permissive to nonpermissive conditions (Barton *et al.*, 1988; Keränen and Kääriäinen, 1979; Sawicki *et al.*, 1981a) and this, together with the presence of the GDD motif found in a number of viral

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polymerases (Kamer and Argos, 1984; Rice *et al.*, 1986), has led to the hypothesis that nsP4 is the RNA polymerase of Sindbis virus. Finally, members of group G (*ts*18 and *ts*7) and group A (*ts*17, *ts*21, and *ts*24) have all been found to map in nsP2 and thus the original complementation found between these groups was intracistronic (Hahn *et al.*, 1989b). From the phenotypes of these various mutants, nsP2 has been implicated in the initiation of 26 S mRNA synthesis, the regulation of minus strand synthesis, and the proteolytic processing of nonstructural polyproteins (Hahn *et al.*, 1989b; Keränen and Kääriäinen, 1979; Sawicki and Sawicki, 1985; Sawicki *et al.*, 1981a,b).

Previously, we have studied the kinetics of synthesis and processing of the nonstructural proteins of Sindbis virus using monospecific antibodies both in infected cells (Hardy and Strauss, 1988) and in cell-free translation systems (Hardy and Strauss, 1989). It seemed important at this point to use these monospecific antibodies to reexamine processing of the nonstructural proteins in cells infected with *ts* mutants whose lesions have been mapped to specific proteins. Furthermore, in the process of mapping, the *ts* lesions have been rescued into an otherwise uniform background, making it possible to rule out the contributions of other changes in the genome to any aberrant processing observed.

MATERIALS AND METHODS

Cell cultures and viruses

The heat-resistant, small plaque strain of Sindbis virus (SIN HRSP) and *ts* mutants *ts*6, *ts*7, *ts*11, *ts*17, *ts*18, *ts*21, and *ts*24 have been described (Burge and Pfefferkorn, 1966a,b; Strauss *et al.*, 1976). Virus stocks were prepared in primary chick embryo fibroblasts (Pierce *et al.*, 1974). The construction of hybrid viruses Toto:*ts*17B1, Toto:*ts*18B1, and Toto:*ts*24B1, in which the *ts* lesions of different mutants were rescued into an otherwise uniform background (Toto1101), and the isolation of same site revertants of the *ts* mutants, have been described (Hahn *et al.*, 1989a,b).

Labeling of infected cells and immunoprecipitation

Duplicate plates (60 or 100 mm) of confluent BHK 21 cells were infected at 30° at a multiplicity of 50 PFU/cell with SIN HRSP, a *ts* mutant, a hybrid virus, or a revertant, as previously described (Hardy and Strauss, 1988). After 70 min the inoculum was removed and replaced with Eagle's minimum essential medium containing 10% dialyzed fetal calf serum, 1 μg/ml actinomycin D, and 1/20 the normal concentration of methionine and incubated at 30°. At 5 hr postinfection this medium was removed and replaced with the same medium prewarmed to 30 or 40° and incubation was continued at 30 or 40°, respectively. One hour later the medium was removed and cells were labeled for 30 min in Eagle's medium lacking methionine but containing 40 μCi/ml of [^{35}S]methionine (>800 Ci/m*M*, Amersham Corp.). The same conditions were used in the pulse–chase experiments with Toto:*ts*17B1 except that chicken embryo fibroblasts were used and 3% dialyzed fetal calf serum was present in the media. At 6 hr postinfection the cells were pulse-labeled for 5 min in Eagle's medium lacking methionine but containing 80 μCi/ml [^{35}S]methionine, and then chased for various times in medium containing 2 μ*M* unlabeled methionine. Whole cell lysates were prepared and immunoprecipitation performed as described (Hardy and Strauss, 1988).

SDS–polyacrylamide gel electrophoresis

SDS–polyacrylamide gel electrophoresis was performed on slab gels containing either 7.5 or 10% (w/v) acrylamide (acrylamide to bisacrylamide ratio 30:0.4 w/w) and the gels were fixed and prepared for fluorography as described (Hardy and Strauss, 1988).

RESULTS

Mutations in nsP1 and nsP4

Cells were infected with *ts*6, whose *ts* lesion is Gly-153 to Glu in nsP4 (Hahn *et al.*, 1989a), with *ts*11, whose lesion is Ala-348 to Thr in nsP1 (Hahn *et al.*, 1989b), or with SIN HRSP, and labeled with [^{35}S]-methionine at 30 or at 40° following a shift from 30°. Labeled proteins were immunoprecipitated with antisera monospecific for nsP1, 2, 3, or 4 and the labeled proteins examined by polyacrylamide gel electrophoresis in SDS (Hardy and Strauss, 1988). In general, there was less label incorporated into nonstructural proteins at 40 than at 30° and processing of the nonstructural polyproteins occurred more rapidly at 40°. Otherwise, the patterns of labeled proteins at the two temperatures were qualitatively similar to one another and the mutant patterns resembled the HRSP patterns (Fig. 1), consistent with previous reports (Keränen and Kääriäinen, 1979; Lopez *et al.*, 1985) and with our previous findings that the deletion of domains in nsP1 or nsP4 does not affect proteolytic processing *in vitro* (Hardy and Strauss, 1989).

We were particularly interested in the results with *ts*11 because Waite (1973) had reported that a polypeptide of approximately 133 kDa that comigrated with the uncleaved structural polyprotein from *ts*13-infected cells accumulated in cells infected with *ts*11 at the nonpermissive temperature. No evidence for such a band was seen in the patterns of the immunoprecipitated nonstructural proteins (Fig. 1C). To determine whether

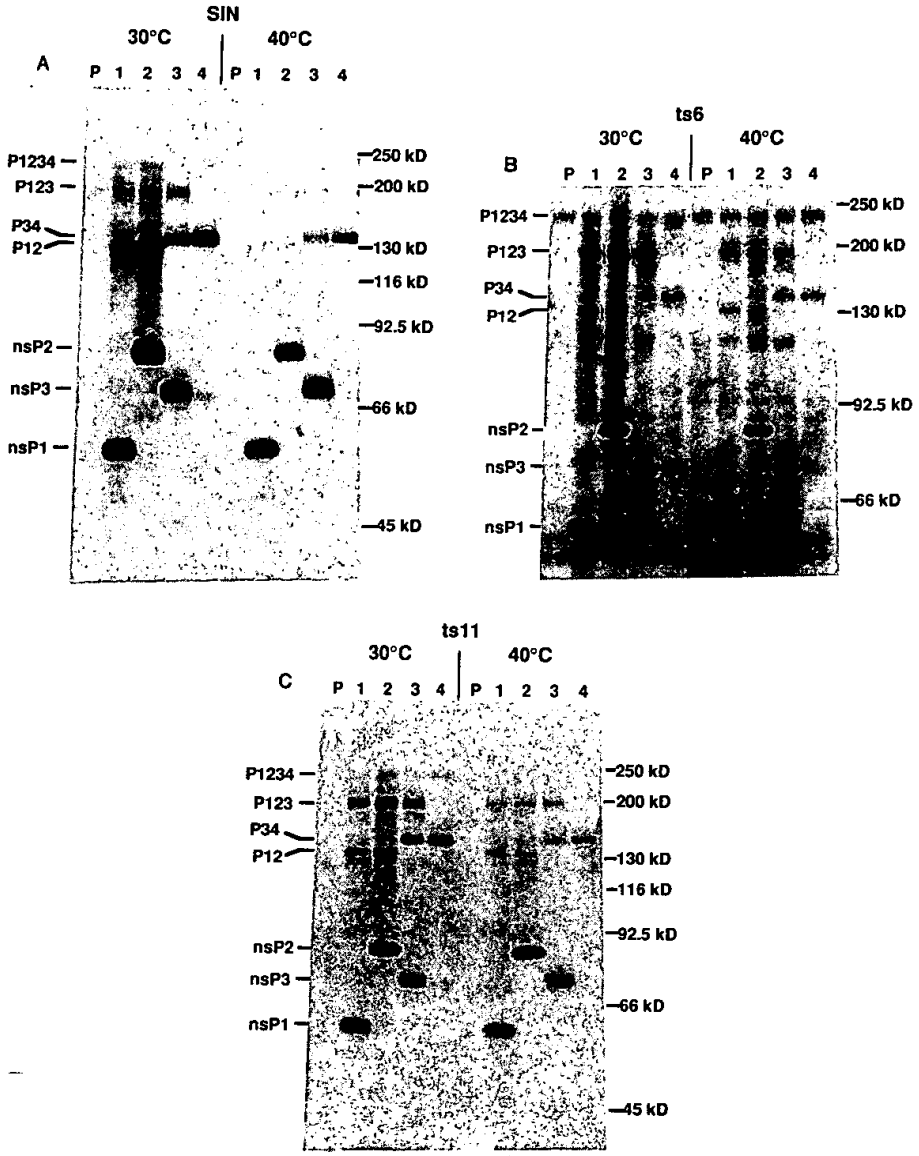

FIG. 1. Polyacrylamide gel electrophoresis of immunoprecipitated nonstructural proteins from cells infected with SIN HRSP, *ts*6, or *ts*11. Duplicate monolayers of BHK cells were infected with 50 PFU/cell for 70 min at 30°. At 5 hr postinfection medium prewarmed to either 30 or 40° was added and the cultures were returned to 30°, or shifted to 40°, respectively. At 6 hr postinfection monolayers were labeled at either 30 or 40° for 30 min in medium containing [^{35}S]methionine, after which the cells were lysed and prepared for immunoprecipitation. Immunoprecipitations were carried out using antiserum specific for each nonstructural protein of Sindbis virus or preimmune serum (P), and the protein patterns were analyzed on 7.5% discontinuous SDS–polyacrylamide gels. (A) SIN HRSP. (B) *ts*6. (C) *ts*11.

this polyprotein might represent an uncleaved structural precursor, we immunoprecipitated proteins from *ts*11-infected cells, labeled at 40° after a shift from 30°, with antisera to the structural proteins C, E1, and PE2 (Rice and Strauss, 1982), and precipitated a polypeptide of approximately 133 kDa. Thus, it appears that *ts*11 also has a lesion in the capsid proteinase which leads to the accumulation of the structural polyprotein, but is not dominant since large amounts of capsid protein and other structural proteins are produced in *ts*11-infected cells at 40° (data not shown).

Mutations in nsP2

Five mutations in nsP2 were examined: *ts*17 (Ala-517 to Thr), *ts*21 (Cys-304 to Tyr), and *ts*24 (Gly-736 to Ser) of complementation group A; and *ts*7 (Asp-522 to Asn) and *ts*18 (Phe-509 to Leu) of group G (*ts*7 also has an unrelated mutation in nsP3, Phe-312 to Ser) (Hahn

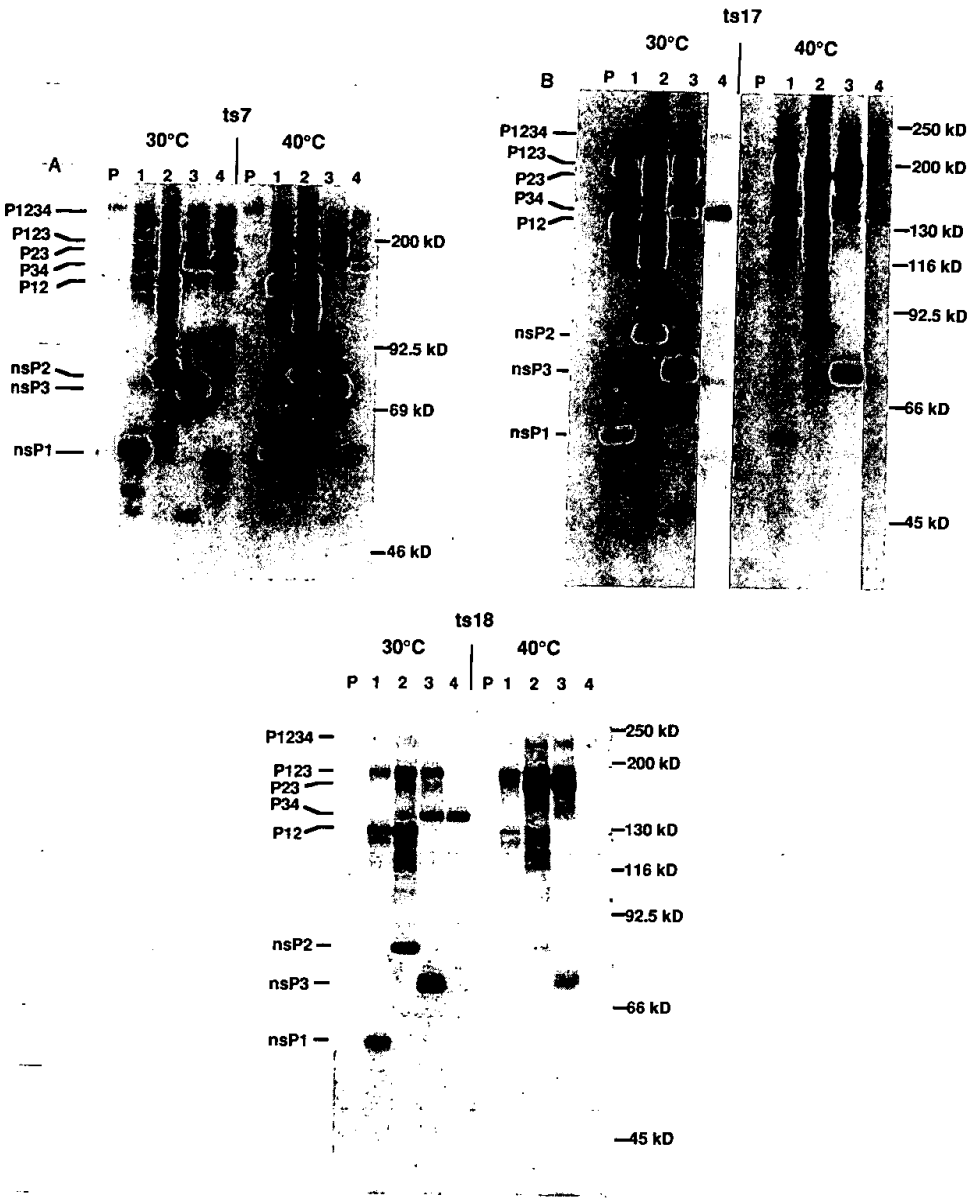

FIG. 2. Polyacrylamide gel electrophoresis of nonstructural proteins immunoprecipitated from cells infected with mutants in nsP2. Cells were infected and labeled, and the proteins were immunoprecipitated as in the legend to Fig. 1. (A) *ts*7. (B) *ts*17. (C) *ts*18.

et al., 1989b). The polyprotein processing pattern for *ts*21 was identical to that for wild-type (data not shown), consistent with our deletion-mapping results (Hardy and Strauss, 1989) that the N-terminal domain of nsP2 is not required for proteolysis. The remaining four mutations, all found within the C-terminal domain of nsP2, affected the polyprotein patterns observed (Fig. 2). In the case of *ts*7 the effects upon the pattern observed were minor (Fig. 2A); the large polyprotein precursors P123 and P1234 were processed, but there was an accumulation of P23, which contains the sequences of nsP2 and nsP3. This polyprotein was not found in SIN HRSP-infected cells (Fig. 1A) in which the dominant pathway is cleavage between nsP2 and nsP3 to produce P12 and nsP3, followed by cleavage of P12 into nsP1 and nsP2 (Hardy and Strauss, 1988).

Mutations in *ts*17, *ts*18, and *ts*24 had a more profound effect upon the polyprotein pattern found at the nonpermissive temperature (Figs. 2B and 2C; the pattern for *ts*24 was very similar to that for *ts*17, data not shown). All three mutants accumulated the high molecular weight precursors P123 and P1234, although

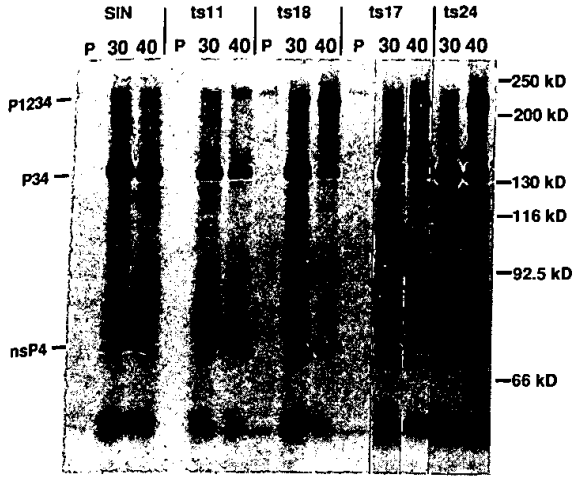

FIG. 3. nsP4 and its precursors in infected cells. For certain *ts* mutants an additional 7.5% discontinuous SDS–polyacrylamide gel was run in which one and one-half times the amount of sample (immunoprecipitated by anti-nsP4 in the above experiment) was loaded per lane. An equivalent amount of lysate from infected cells labeled at 40° and immunoprecipitated with preimmune serum was run alongside to serve as a control. Because of the small quantity of nsP4 present, a longer exposure time was used than for the autoradiograms shown in Figs. 1 and 2.

the analysis of P1234 is complicated by the fact that a host band that comigrates with it precipitates nonspecifically under some conditions (see Hardy and Strauss, 1988). These three mutants also demonstrated reduced amounts of processed polyproteins P12 and P34, as well as reduced amounts of mature nonstructural proteins, in comparison with SIN HRSP-infected cells at 40°. Furthermore, these mutants accumulated large amounts of polyprotein P23 at 40° (this polyprotein of ~175 kDa is not well resolved from P123 in the pattern of *ts*17 because of overexposure necessary to bring out the fainter bands), suggesting that cleavage of the 2–3 site was most seriously impaired in these mutants.

It is noteworthy that the amounts of protein present in the mutant patterns at 40° were not stoichiometric. Although large amounts of P23 were found, little nsP1 was detected. Furthermore, although significant amounts of nsP3 accumulated, more so in *ts*24 and *ts*17 than in *ts*18, corresponding amounts of nsP2 were not found. It appears that both nsP1 and nsP2 were unstable and were degraded under these conditions or were never produced. Since the *ts* lesion is in nsP2, abnormal folding of this protein could lead to its instability, but the situation for nsP1 is less clear. Two possibilities are that the abnormal proteinase leads in some way to more rapid turnover of nsP1 and nsP2, or that nsP1 and nsP2 function as a complex and when these proteins are produced in an abnormal processing pathway they are unstable (see below).

From the large amounts of P23 present and the significant amounts of nsP3 present it seems clear that the mutant proteinase was still able to cleave both the 1–2 and the 2–3 bonds at the nonpermissive temperature, although cleavage of the 2–3 bond was less complete than that of the 1–2 bond. To examine cleavage of the 3–4 bond more carefully, a larger amount of labeled material was analyzed and longer exposure times were used (Fig. 3). For SIN HRSP and the *ts* mutants that are not defective in processing, more mature nsP4 and its precursors, P34 and P1234, were present at 30° than at 40°. However, in mutants that are *ts* for processing, *ts*17, *ts*18, and *ts*24, P1234 accumulated and reduced amounts of P34 were produced at 40°. The reduction in the amount of P34 at 40° was particularly striking in the *ts*17 pattern, which will be addressed in more detail below. Despite the accumulation of P1234, the relative amount of nsP4 present at 30 or at 40° for these mutants was similar to that found in SIN HRSP. This suggests that the concentration of nsP4 is regulated by something in addition to the rate of its production by proteolytic cleavage.

Nonstructural protein processing by rescued mutants

Previously, the mutations in *ts*17, *ts*18, and *ts*24 were rescued into a uniform background, Toto1101, and these hybrid viruses were characterized, as were same site revertants of these mutants (Hahn *et al.*, 1989b). In order to rule out the possible contribution of unmapped lesions in the viral genome to the aberrant processing patterns we observed, we also examined the polyproteins synthesized and processed by these hybrid viruses and revertant viruses (Fig. 4). The results were essentially identical with those obtained with the parental *ts* mutants. In Toto:*ts*17B1, Toto:*ts*18B1, and Toto:*ts*24B1, large amounts of P123 and P23 were present at 40°, and in *ts*17B1 and *ts*24B1 larger than wild-type amounts of P1234 were present, whereas nsP1 and nsP2 were underrepresented. It is also clear that nsP1 and nsP2 were underrepresented, and detectable amounts of P23 were present, at 30 as well as at 40° in *ts*18B1 and *ts*24B1. Revertant viruses *ts*17R, *ts*18R, or *ts*24R gave patterns similar to SIN HRSP. Thus it is clear that the aberrant polyprotein processing observed results from the *ts* lesions mapped in nsP2.

Stability of nonstructural proteins in Toto:*ts*17-infected cells

Pulse–chase experiments to examine the processing and degradation of the nonstructural proteins are difficult to interpret because the time required to synthesize a polypeptide chain (~15 min) is fairly long in comparison with the time required to cleave the precur-

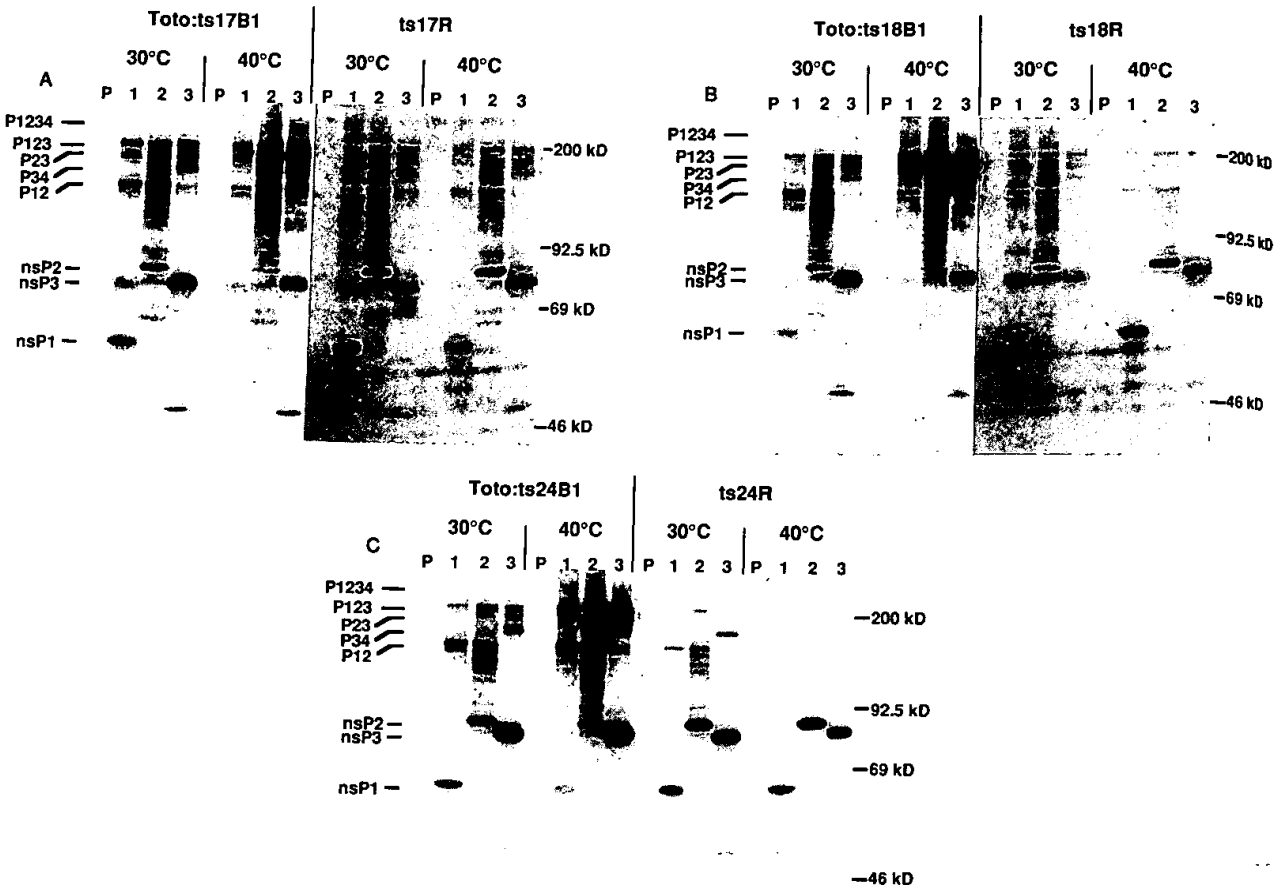

FIG. 4. Nonstructural proteins immunoprecipitated from cells infected with the rescued mutants. BHK cells were infected, labeled, and harvested under conditions identical to those used for the parental viruses. Preimmune serum or antiserum to each of the nonstructural proteins of Sindbis was used for the immunoprecipitations and the labeled proteins were resolved on 10% SDS–polyacrylamide gels. (A) Toto:*ts*17B1 or *ts*17R. (B) Toto:*ts*18B1 or *ts*18R. (C) Toto:*ts*24B1 or *ts*24R.

sors (Hardy and Strauss, 1988) and with the apparent time required to degrade nsP1 or nsP2 in the experiments above (Figs. 2 and 4). Nevertheless, we wished to perform a pulse–chase experiment in an attempt to examine the apparent instability of nsP1 and nsP2 in cells infected with the proteinase-defective mutants upon shift to 40°. Furthermore, such an experiment would allow us to examine more closely the instability of P34 in *ts*17-infected cells (Fig. 3). For these reasons cells were infected with Toto:*ts*17B1 at 30°, shifted to 40° at 5 hr, and 1 hr after the shift labeled for 5 min with [^{35}S]methionine. Control cells were maintained at 30° throughout. After varying periods of chase, the cells were lysed and immunoprecipitated with each of the four monospecific antisera. The results are shown in Fig. 5.

In this pulse–chase experiment, the amount of incorporation and the rate of elongation was greater at 40 than at 30°. Incorporation plateaued at 15–30 min, after which time the polyproteins were chased, and largely disappeared over the 60 min of chase. Although mature nonstructural proteins and P34 accumulated at 30°, at 40° the loss of polyprotein precursors was not accompanied by a corresponding increase in mature nonstructural proteins. Most striking were the reduced levels of mature nsP1 and nsP2 present at 40° (Fig. 5A). The instability of nsP1 is readily apparent in Fig. 5A where, in addition to nsP1, there appears a smaller, diffuse band which probably represents a degradation product. nsP2, on the other hand, never appears to accumulate at 40°. This finding suggests either that it is degraded at a faster rate than it is produced by translation and cleavage of the precursors, or that the precursors themselves are unstable and are degraded nonspecifically before nsP2 is cleaved. This experiment seems to indicate that nsP3 is also somewhat unstable at 40° and decreases in amount between 30 and 60 min of chase (Fig. 5B). Note that after 60 min of chase

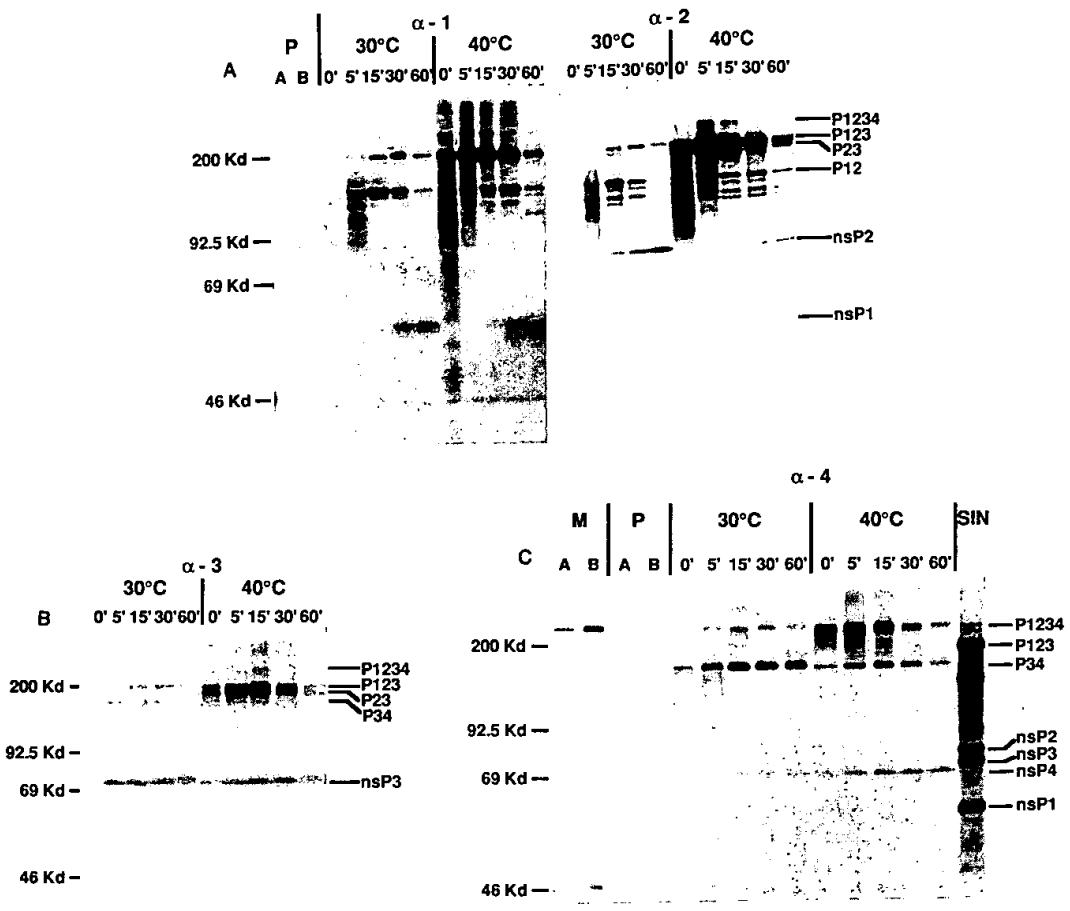

FIG. 5. Pulse-chase experiment in Toto:*ts*17-infected cells. Duplicate monolayers of chicken embryo fibroblasts were infected with Toto:*ts*17B1, using the same conditions as in the legend to Fig. 1. At 6 hr postinfection the cells were pulse-labeled with [^{35}S]methionine for 5 min at either 30 or 40° after shift from 30° at 5 hr, and then chased for various times in the presence of excess unlabeled methionine. Whole cell lysates were prepared and immunoprecipitated with antisera to the four nonstructural proteins (designated α-1, α-2, etc.). As a control, preimmune serum (P) was also used to immunoprecipitate the 30-min time points at either 30 (A) or 40° (B). The precipitated products were analyzed on 10% discontinuous SDS-polyacrylamide gels.

at 30°, nsP3 migrates somewhat more slowly, as has been described (Hardy and Strauss, 1988). The shift in mobility after 60 min at 40° does not seem as marked. From the fact that nsP1, nsP2, nsP3, and nsP4 are all produced at 40° albeit in small amounts, we conclude once again that cleavage occurs at all three sites but with slow kinetics, particularly at the 2–3 site which results in the accumulation of P23.

The results after immunoprecipitation with anti-nsP4 clearly show that at 40° P34 is unstable, accumulating for the first 15 min of chase (through continued polypeptide chain elongation followed by processing), then disappearing over the next 45 min of chase (Fig. 5C). To examine the processing and accumulation of nsP4 and of P34 more precisely, the amounts of P1234, P34, and nsP4 in this experiment were quantitated and the results are plotted in Fig. 6. In this figure the amounts of radioactivity in each protein have been normalized for the number of methionines such that only the label corresponding to nsP4 in any protein is plotted. Thus, for example, nsP4 has 16 methionines and P34 has 27; the amount of label in P34 was multiplied by 16/27 before plotting in Fig. 6. At 30° P34 accumulated in large amounts and was stable during the chase, while P1234 accumulated only during the first 15 min and then disappeared. nsP4 accumulated gradually and was present in only about 10% of the amount in P34 over the course of this experiment. The results upon chase at 40° were quite different. P1234 was found in relatively much larger amounts early in the chase, and then decayed rapidly. P34 was also unstable during the chase, decaying with a half-life of about 30 min. nsP4 accumulated in relatively larger amounts such that after 60 min of chase it was present in greater amounts than P34. The possible significance of this is discussed below. Note also that the amounts of nsP4 found at 40°

are less that would be predicted from the amounts in P1234 and P34 that disappear during the chase, indicating that nsP4, although it accumulated in the experiment, is unstable.

DISCUSSION

The nsP2 proteinase

We have examined the processing of the nonstructural polyproteins in cells infected with a number of *ts* mutants of Sindbis virus whose lesions have been mapped to specific amino acids in the nonstructural proteins. The use of monospecific antisera allowed the identification of polyproteins present on the basis of the sequences they contain rather than simply on the basis of molecular weight and therefore allowed the precise identification of each polyprotein. Furthermore, by using mutants whose lesions had been mapped, and in the case of mutants which affect polyprotein processing severely, the use of hybrid viruses in which these mutations had been transferred into an otherwise uniform background, we have been able to define precisely the *ts* lesions that result in aberrant processing. Lesions in nsP1, nsP4, or in the N terminus of nsP2 did not affect processing, whereas four mutations in the C terminus of nsP2 all affected processing to a greater or lesser extent, consistent with our deletion mapping studies that the nonstructural proteinase is in the C terminus of nsP2 (Hardy and Strauss, 1989).

Bracha *et al.* (1976) reported that the cleavage of the precursor P123 (called p200 in their paper) was *ts* in *ts*21-infected cells. We found that polyprotein processing in *ts*21-infected cells was identical to that in SIN HRSP-infected cells, in agreement with the results of Keränen and Kääriäinen (1979) and of Sawicki and Sawicki (1985). We have also shown that the polyprotein that accumulates in *ts*11-infected cells at the nonpermissive temperature first reported by Waite (1973) results from the failure of structural polyprotein processing rather than of nonstructural polyprotein processing.

Aberrant processing by the mutant proteinases

In all four mutants examined that were *ts* in processing, the processing pathways were changed quantitatively, if not qualitatively, at 40°. Analogous results have been found in poliovirus where *ts* mutations in the 3C proteinase can result in altered cleavage specificities (Dewalt *et al.*, 1990). In the Sindbis virus mutants, the abundance of P23, a precursor not normally seen in wild-type-infected cells, suggests that cleavage of the 2–3 bond is more affected than the cleavages at the other cleavage sites. However, for those mutants most severely defective in processing, namely *ts*17, *ts*18, and *ts*24, significant amounts of both P123 and P1234 also accumulated at the nonpermissive temperature, indicating that the rate of processing at the other sites was reduced as well. Normally cleavage of P1234 appears to occur very rapidly, whether in infected cells or in cell-free translation systems, probably while the polypeptide is nascent. Cleavage occurs either at the 3–4 site, leading to the production of P123 and nsP4, or at the 2–3 site, leading to the production of P12 and P34. Early in infection the former pathway predominates, whereas later in infection the latter pathway predominates (Hardy and Strauss, 1988; 1989; Shirako and Strauss, 1990; de Groot, Hardy, Shirako, and Strauss, submitted for publication). Thus, the processing pathways in these mutants upon shift to 40° recall the situation that predominates early in infection.

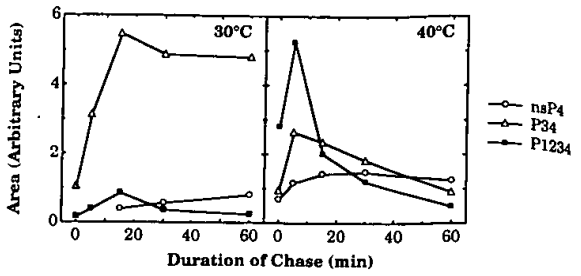

FIG. 6. Quantitation of the amounts of nsP4 and its precursors in Toto:*ts*17B1-infected cells. The autoradiograph in Fig. 5C was densitometered and the areas corresponding to nsP4, P34, and P1234 were determined. The area in P34 was multiplied by 16/27 and that in P1234 by 16/57 so that only the radioactivity in nsP4 is plotted for any protein. The normalized radioactivity is plotted in arbitrary units of area.

We have found that polyproteins containing nsP2 are active proteinases that act primarily *in trans,* and that different polyproteins differ in their cleavage site specificity with respect to the 1–2, 2–3, and 3–4 bonds (Hardy and Strauss, 1988, 1989; Shirako and Strauss, 1990; de Groot, Hardy, Shirako, and Strauss, submitted for publication). Thus, one possible explanation for the observation that cleavage of the 2–3 site is most inhibited in these mutants is that the misfolding induced by the *ts* lesion affects the various polyprotein proteinases differently such that those with a preference for the 2–3 bond are most affected. A second related possibility is that the misfolding induces a change in specificity of the polyprotein proteinases. Specifically, for example, the Sindbis polyprotein when first synthesized is able to cleave the 1–2 and the 3–4 bond but not the 2–3 bond. Only after cleavage of the 1–2 bond can the resulting polypeptide cleave the 2–3 bond. It is unknown why the cleaved polypeptide has an altered specificity for the cleavage sites, but if a shift in conformation of the proteinase were involved, this shift might not occur in the *ts* proteinases. Finally, the

difference in processing pathways may be related to an inactivation or a reduction of proteinase activity upon shift; as noted above the pattern upon shift in these experiments recalls that seen early in infection.

The shift in the processing pathway at 40° in the mutants also seems to have effects upon the stability of nsP1 and nsP2, which appear to be unstable in cells infected with *ts*17, *ts*18, and *ts*24. Late in infection by wild-type Sindbis virus, the predominant processing pathway to produce nsP1 and nsP2 is autoproteolysis of the P12 precursor (Hardy and Strauss, 1989). There is reason to believe that nsP1 and nsP2 might interact with one another to form a functional complex, because several plant viruses, including tobacco mosaic virus, possess proteins that are homologous to nsP1 and nsP2, but in these plant viruses one polypeptide contains both the nsP1 and nsP2 domains (Ahlquist *et al.*, 1985). It is possible that free nsP1, not complexed with nsP2, or free nsP2, might be unstable. Thus when nsP1 and nsP2 are produced by autoproteolysis of P12 they might remain associated in a stable functional complex, but when nsP1 is released from P123 by cleavage of the 1–2 bond, or when nsP2 is released from P23 by cleavage of the 2–3 bond, one or both of the separated polypeptides might be unstable. It is also possible, however, that the apparent instability results from aberrant processing or degradation of the polyprotein precursors.

Regulation of RNA synthesis

As described in the Introduction, nsP4 is believed to contain the RNA polymerase activity of Sindbis virus, and readthrough of an opal stop codon is required for its translation. It is thus of particular interest that the levels of nsP4 produced by mutants *ts*17, *ts*18, and *ts*24 at the nonpermissive temperature were similar to that in wild-type virus, despite quite different amounts of P1234 and of P34 in these mutants and wild type. The pulse–chase experiment demonstrated that the amount of nsP4 in *ts*17-infected cells remained almost constant despite the disappearance of large amounts of P1234 and P34. This could imply that the concentration of nsP4 is maintained at some steady-state level, such that any excess nsP4 produced is degraded, perhaps by the proteinase itself (see also Hardy and Strauss, 1988). In cells infected with the closely related Semliki Forest virus, in which no opal codon is present between nsP3 and nsP4 and nsP4-containing polypeptides are produced in four- to fivefold greater quantities than in Sindbis virus-infected cells, nsP4 also appears to be less stable than the other three nonstructural proteins. This difference in stability is even more pronounced in cells infected with *ts*1, a mutant of Semliki Forest virus which overproduces the nonstructural proteins at the nonpermissive temperature (Keränen and Ruohonen, 1983). These data suggest that nsP4 concentrations are also regulated in Semliki Forest virus even though the opal termination codon is not present.

A second point of interest concerns the ratio of P34 to nsP4 in *ts*17. We have postulated that P34 is the RNA polymerase for subgenomic RNA synthesis and that nsP4 is the RNA polymerase for minus strand synthesis. We have found that at 3–4 hr after infection there is an increase in the amount of P34 relative to nsP4, and postulated that this change is responsible for the shutdown of minus strand synthesis and the shift to production of 26 S subgenomic RNA that occurs in wild-type-infected cells at this time (de Groot, Hardy, Shirako, and Strauss, submitted for publication). Upon shift to 40°, P34 is unstable in *ts*17-infected cells, in contrast to the situation in wild-type infection or in the other mutants examined, such that the ratio P34/nsP4 decreases profoundly. Our model would then predict that 26 S RNA synthesis would cease and that minus strand synthesis would resume upon shift (i.e., that synthesis of 26 S RNA and regulation of minus strand synthesis are *ts* in this mutant). This has been reported to be the case for *ts*17 by Sawicki and colleagues (Sawicki *et al.*, 1981a,b; Sawicki and Sawicki, 1985, 1986). In *ts*17 the single mutation mapped by Hahn *et al.* (1989b) to the nsP2 proteinase also appears to be responsible for the *ts* lesion in 26 S RNA synthesis and regulation of minus strand synthesis, because a revertant to temperature insensitivity for growth also reverted to temperature insensitivity of RNA synthesis (Sawicki and Sawicki, 1985, 1986). The instability of P34 in *ts*17 presumably arises from the altered processing pathways in the infected cell such that large amounts of active proteinases capable of cleaving P34 are present (P123, P23, P234), or from altered cleavage specificities in the *ts* proteinase. In any event, the results are in accord with the model that control of the virus RNA synthesis is accomplished by differential cleavage pathways controlled by different cleavage specificities of the polyprotein precursors. It is of interest, however, that Sawicki *et al.* (1990) have reported that in a different mutant, *ts*24, a mutation in nsP4 results in *ts* regulation of minus strand synthesis. This nsP4 mutation does not render the virus *ts* for growth and is distinct from the *ts* lesion in the proteinase reported upon here, which as noted did not result in an unstable P34 at 40°. Thus in this nsP4 mutant failure to regulate minus strand synthesis appears to result from altered affinities of P34 or nsP4 for the RNA templates rather than from alterations in the ratios of these proteins.

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